Supporting Information

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SI Materials and Methods

hMSC Cell Culture. Frozen vials containing about 1 million passage 1 hMSCs from bone marrow were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (formerly http://www.som.tulane.edu/gene_therapy/distribute.shtml; currently http://medicine.tamhsc.edu/irm/msc-distribution.html). hMSCs were isolated from 1–4 mL bone marrow aspirates of the iliac crest in normal adult donors. Nucleated cells, obtained by density gradient centrifugation (Ficoll-Paque; GE Healthcare), were resuspended in CCM: α-MEM (Gibco), 17% FBS (Atlanta Biologicals), 100 units/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 2 mM L-glutamine (Gibco), seeded in 175 cm^2 flasks (Nunc), and subsequently cultured at 37 °C in a humidified atmosphere with 5% CO_2. After 24 h, nonadherent cells were discarded. Adherent cells were incubated 4–11 d until approximately 70% confluent, harvested with 0.25% trypsin and 1 mM EDTA (Gibco) for 5 min at 37 °C, and replated at 50 cells/cm^2 in an intercommunicating system of culture flasks (Nunc). The cells were incubated for 7–12 d until approximately 70% confluent, harvested with trypsin/EDTA, and frozen as passage 1 cells in α-MEM containing 30% FBS and 5% DMSO (Sigma). A frozen vial of passage 1 hMSCs (donor 1, 7064 L; donor 2, 7068 L) was thawed, resuspended in CCM, and plated in a 152-cm^2 culture dish (Corning). After 24 h, adherent cells were harvested using trypsin/EDTA, plated at 100 cells/cm^2, and expanded for 7 d before freezing. In this study, passage 1 or 2 frozen hMSCs were recovered, seeded at 100 cells/cm^2 24 h later, and grown 7–8 d in CCM for various assays.

Spheroid Generation and Dissociation. To generate spheroids, hMSCs were plated as hanging drops on an inverted culture dish lid in 35 μL of CCM at 10,000–250,000 cells/drop. The lid was flipped and placed on a culture dish containing PBS (Gibco) to prevent evaporation. Hanging drop cultures were grown at 37 °C up to 4 d in a humidified atmosphere with 5% CO_2. Spheroid generation in hanging drops was captured using a Photometrics Coolsnap HQ2 camera mounted on a Nikon Eclipse Ti-E inverted microscope containing a temperature controlled environmental chamber. To collect spheroids, drops were harvested using a cell lifter, transferred to a 15 or 50 mL conical tube (Falcon), washed with PBS, and centrifuged at $453 \times g$ for 5–10 min. To obtain spheroid derived cells, spheroids were incubated with trypsin/EDTA at 37 °C for 5–30 min (5 min for 10k, 10 min for 25k, 20 min for 100k, and 30 min for 250k spheroids), while pipetting every 2–3 min. When no cell aggregates were visible, spheroid derived cells were collected by centrifugation at $453 \times g$ for 5–10 min to be used in described assays.

Histology. hMSC spheroids were collected with cell lifter (Corning), transferred into a 15-mL conical tube, washed twice with PBS, and fixed with 2% paraformaldehyde (USB Corporation) in PBS for 15 min at room temperature. Fixed spheroids were washed twice with PBS, centrifuged at $500 \times g$ for 10 min, and incubated at 4 °C overnight in 500 μL of 30% sucrose solution (Sigma) in 0.1 M phosphate buffer (Sigma). After incubation, 800 μL of 30% OCT (Sakura Finetek) in sucrose solution was added gently and the suspension was transferred into a histology mold. The mold was frozen in isopentane (Sigma) chilled by liquid nitrogen and stored at −80 °C. Cryosections (6 μm) were prepared with a Microm HM560 cryostat. For H&E staining, slides were first incubated at room temperature for 10–15 min, fixed in 4% PFA for 15 min, and washed twice with deionized water. Rehydration was performed by incubating the samples in 100% ethanol (EMD Chemical) for 5 min, 95% ethanol for 2 min, 70% ethanol for 2 min, and deionized water twice for 5 min. The slides were stained with Mayer's Hematoxylin (Electron Microscopy Science) for 15 min, rinsed with deionized water, incubated with Scott's Tap Water Substitute (Ricca Chemical Company) for 2 min, rinsed with deionized water, washed with warm tap water for 20 min, rinsed with deionized water, immersed in 95% ethanol for 1 min, and stained with Eosin Y (Mallinckrodt Baker) for 1 min. Slides were then dehydrated by immersion in 95% ethanol for 1 min, 95% ethanol for 5 min, 100% ethanol for 5 min, and xylene (EMD Chemical) for 5 min. Samples were air-dried and overlaid with coverslips for examination. Mounting media (VECTA Mount; Vector Laboratories) was used to preserve staining. Images were acquired on a Nikon Eclipse 80i upright microscope and processed using NiS Elements AR 3.0 software (Nikon).

Real-Time RT PCR Assays. Total RNA was isolated from monolayer and spheroid hMSCs using RNeasy Mini Kit (Qiagen) with DNase (RNase-Free DNase Set; Qiagen) digestion step. RNA was converted into cDNA with High-Capacity cDNA RT Kit (Applied Biosystems). Real-time RT PCR was performed in triplicate for 18 s, TSG-6 (TNFAIP6), STC1, LIF, IL-24, TRAIL, CXCR4, and DKK1 using Taqman Gene Expression Assays (Applied Biosystems). A total of 15–60 ng of cDNA was used for each 20-μL reaction. Thermal cycling was performed with 7900HT System (Applied Biosystems) by incubating the reactions at 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Data were analyzed with Sequence Detection Software V2.3 (Applied Biosystems) and relative quantities (RQs) were calculated with comparative C_T method using RQ Manager V1.2 (Applied Biosystems). If no amplification occurred, C_T value of 35 was used in calculating the RQs.

Viability Assays. Spheroid viability was measured by flow cytometry (FC500; Beckman Coulter) using Annexin V-FITC apoptosis detection kit (Sigma) per manufacturer's instructions. Spheroids were collected and washed in PBS, followed by debris removal with a 40-μm cell strainer (Fisher). Spheroids were then transferred into a sterile centrifuge tube by inversion of the strainer and subsequently pelleted by centrifugation at $453 \times g$ for 5 min. The supernatant was aspirated and the spheroids dissociated in a six-well plate (Corning) at 37 °C using 2–3 mL trypsin/EDTA. After 5–30 min, the digest was neutralized with FBS, filtered through a 40 μm cell strainer to remove nondissociated particles, and centrifuged at $453 \times g$ for 5–10 min to acquire a cell pellet. Approximately 200,000 hMSCs derived from monolayer cultures or spheroids were incubated for 10 min with 0.5 μg/mL annexin V-FITC and 2 μg/mL PI in 400 μL of 1× binding buffer (10 mM Hepes, 0.14 M NaCl, 2.5 mM $CaCl_2$). The cells were immediately placed on ice and analyzed. Cell fragments were removed by morphological gating. Cells negative for annexin V-FITC and PI were considered viable, annexin V-FITC positive and PI negative considered apoptotic, and annexin V-FITC positive and PI positive considered necrotic.

Cell Cycle Analysis. The cell cycle distribution in spheroids and monolayer cultures was determined by analyzing DNA content of permeabilized hMSCs labeled with propidium iodide (Sigma). hMSCs derived from monolayer or spheroid cultures were resuspended in 1 mL of ice cold PBS containing 2% FBS followed by fixing with 3 mL cold absolute ethanol added dropwise while

vortexing. The cells were incubated for 2–4 h in 4 mL of 75% ethanol to complete fixation, washed 3 times in PBS, then pelleted by centrifugation at 800 × *g* for 10 min. Cells were incubated with 7 U/mL RNase A (Qiagen) in 1 mL of PBS at room temperature. After 1 h, 50 μg/mL propidium iodide was added and the cells were incubated overnight at 4 °C. DNA content was measured with a flow cytometer and data analyzed using MultiCycle software (Phoenix Flow Systems).

Cell Surface Protein Detection. To analyze cell surface markers, hMSCs resuspended at 3.0×10^6 cells/mL in αMEM containing 2% FBS were labeled with the antibodies described (Table S1) for 40 min on ice. The cells were washed 3 times with PBS and surface expression of proteins was determined with a flow cytometer.

Spheroid-Derived Cell Sizing. The size of hMSCs derived from adherent monolayers, or from dissociated spheroids suspended for 3 d in hanging drops at 10,000, 25,000, 100,000, and 250,000 cells/drop, was determined by microscopy and flow cytometry. For microscopic analysis, the cells were transferred into chambers of a Neubauer improved disposable hemocytometer and images captured on a Nikon Eclipse Ti-S inverted microscope using a Ds-Fi1 camera (Nikon). Cell diameter of more than 50 cells per group was subsequently determined using NIS-Elements AR 3.0 software. For flow cytometric analysis of cell size, 2.0×10^5 hMSCs were resuspended in 400 μL αMEM containing 2% FBS then incubated for 20 min with 100 nM of the live cell viability dye calcein AM (Molecular Probes) and 10 min with 2 μg/mL of the dead cell nuclear label 7AAD (Sigma). Cell sizes were estimated from the viable population (Calcein$^+$/7AAD$^-$) by comparing forward scatter (FS) properties of the cells and beads with a known diameter of 3, 7, 15, or 25 μm. Brackets were subsequently applied to the scatter plot at locations corresponding to the respective bead size. Gates established based on bead size FS were used to group the cells into five populations (<3 μm, 3–7 μm, 7–15 μm, 15–25 μm, and >25 μm).

Intravenous Infusion of hMSCs. Male immunodeficient NOD/scid mice (NOD.CB17-Prkdcscid/J; The Jackson Laboratory), 7–8 wk of age, housed on a 12 h light/dark cycle, were used to study the relative tissue distribution of the i.v. infused hMSCs. All animal procedures were performed with approval by the Animal Care and Use Committee of Texas A&M Health Science Center and in accordance with guidelines set forth by the National Institutes of Health. Mice were anesthetized by i.p. injection of a mixture of ketamine (91 mg/kg) and xylazine (9 mg/kg). Total of 10^6 monolayer or spheroid derived hMSCs suspended in 150 μL of HBSS were infused slowly into a tail vein. Mice were anesthetized 15 min later with ketamine/xylazine (90 mg/kg and 9 mg/kg) and euthanized by exsanguination. Heart, lung, liver, spleen, and kidneys were isolated by dissection and stored at −80 °C for further analysis.

Isolation of Genomic DNA. After thawing the tissues, 5 mL of 10 mM Tris HCl (pH 8.0) containing 20 μL proteinase K (10 mg/mL), 0.1 mM EDTA (pH 8.0), 0.5% SDS, and 20 μg/mL RNase A was added to each sample. Samples were homogenized (PowerGen Model 125 Homogenizer; Fisher Scientific) and incubated at 50 °C overnight on a shaker at 200 rpm. DNA was extracted by mixing 0.5 mL of sample with 0.5 mL phenol/chloroform solution (pH 6.7) followed by centrifugation at 15,300 × *g* for 5 min in 2 mL phase lock gel tubes (Phase Lock Gel; Eppendorf/Brinkmann Instruments). To precipitate the DNA, 1/2 volume of 2.5 M ammonium acetate, and the same volume of 100% ethanol was added, followed by overnight incubation at 4 °C. The precipitates were washed with ice cold 75% ethanol and resuspended into sterile water.

Real-Time PCR Assays for Alu Sequences. Real-time PCR assays for Alu sequences were performed in 50 μL containing 25 μL Taqman Universal PCR Master Mix (Applied Biosystems), 900 nM each of the forward and reverse primers, 250 nM TaqMan probe, and 200 ng of genomic DNA. Reactions were incubated at 50 °C for 2 min and at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Real-time PCR assays for human and mouse GAPDH genes were performed in 50 μL containing 25 μL SYBR Green Master Mix (Applied Biosystems), 200 nM each of the forward and reverse primers and 200 ng of genomic DNA. All real-time PCR assays were performed in duplicate or triplicate and average values are presented. The final value for total DNA in the sample was corrected by parallel real-time PCR assays with primers that amplified both the human and mouse GAPDH genes.

Differentiation Assays. hMSCs derived from high density monolayer (5,000 cells/cm^2) or hanging drop cultures (25,000 cells/drop), grown for 3 d, were seeded at low density (100 cells/cm^2) on six-well dishes and were grown until 80–90% confluent. To induce adipogenesis, hMSCs were cultured in CCM supplemented with 500 nM dexamethasone (Sigma), 500 nM isobutylmethylxanthine (Sigma), and 50 μM indomethacin (Sigma) for 14 d with medium changes every 3–4 d. To induce osteogenesis, hMSCs were cultured in CCM supplemented with 10 nM dexamethasone, 10 mM β-glycerolphosphate (Sigma), and 50 μM Ascorbate-2-phosphate (Sigma) for 14 d with medium changes every 3–4 d. Parallel control cultures were maintained in CCM for 14 d with medium changes every 3–4 d. All wells were washed with PBS and fixed with 10% neutral buffered formalin (Sigma) for 1 h. The adipogenic differentiation and control wells were washed with PBS and stained with 0.6% Oil-Red-O (Sigma) solution in 60% isopropanol (Sigma) and 40% PBS for 20 min followed by washing with PBS. The osteogenic differentiation and control wells were washed with deionized water and stained with 1% Alizarin Red S (Sigma) solution, pH 4.2, in water for 20 min followed by washing with water. Images were captured on a Nikon Eclipse Ti-S inverted microscope.

Growth Curves. hMSCs derived from high density monolayer or hanging drop cultures were seeded at 100 cells/cm^2 in 55-cm^2 dishes (Corning) in quadruplicate and cultured for 7 d in CCM with medium changes every 3 d. After 7 d, cells were lifted with trypsin/EDTA, counted with hemocytometer, and replated. The process was repeated until cells reached senescence.

CFU-F assays. hMSCs derived from high density monolayer or hanging drop cultures were seeded onto 55 cm^2 dishes at 1.5 cells/cm^2 in quadruplicate and cultured for 14 d in CCM. Medium was changed every 3–4 d. After 14 d, the plates were stained with 3% crystal violet (Sigma) in 100% methanol (Sigma) for 5 min, washed with water, and air-dried. Plates were scanned on an EPSON Perfection 4490 Photo scanner and images were processed with Adobe Photoshop CS3.

Microarrays. hMSCs from two donors grown at low density for 7 d and at high density or in hanging drops for 3 d were harvested for total RNA. A total of 2 μg of RNA from each sample was applied for microarrays using Whole Transcript Sense Target Labeling Assay protocol (Affymetrix) according to manufacturer's directions. Briefly, to minimize the background and thereby increasing the array detection sensitivity and specificity, rRNA reduction was performed for samples containing the Poly-A RNA controls (GeneChip Eukaryotic Poly-A RNA-Control Kit; Affymetrix) using the RiboMinus Transcriptome Isolation Kit (Invitrogen) with Magna-Sep Magnetic Particle Separator (Invitrogen) and Betaine (Sigma). RNA was concentrated with GeneChip IVT cRNA Cleanup Kit (Affymetrix) and used to prepare double

stranded cDNA with GeneChip WT cDNA Synthesis Kit (Affymetrix). Generated cDNA was used to produce cRNA with GeneChip WT cDNA Amplification Kit (Affymetrix), followed by cleanup with GeneChip Sample Cleanup Module (Affymetrix). The amount of cRNA was determined with spectrophotometer (SmartSpec Plus; Bio Rad) and 10 μg was used to generate cDNA with GeneChip WT cDNA Synthesis Kit (Affymetrix) followed by cRNA hydrolysis and cleanup of single-stranded cDNA with GeneChip Sample Cleanup Module. The amount of cDNA was determined with spectrophotometer and 5.5 μg was used for fragmentation with GeneChip WT Terminal Labeling Kit (Affymetrix). The fragmented cDNA was labeled using GeneChip WT Terminal Labeling Kit and hybridized (GeneChip Hybridization Oven 640; Affymetrix) on Human Exon 1.0 ST arrays (Affymetrix) using GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). Arrays were washed and stained (GeneChip Fluidics Station 450; Affymetrix) using GeneChip Hybridization, Wash, and Stain Kit followed by scanning with GeneChip Scanner (Affymetrix). Data were normalized using robust multiarray (RMA) algorithm and gene level analysis was performed with Partek Genomics Suite 6.4 (Partek). Genes that were either up- or down-regulated in spheroids at least twofold, compared with their monolayer counterparts, were used in hierarchical clustering. Significant Gene Ontology terms for up- and down-regulated genes in spheroids were determined using the Partek software. The raw microarray data files will be available at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/).

Analysis of hMSC-Secreted Soluble Antiinflammatory Factors. For TSG-6, STC-1, and LIF ELISAs, monolayer, spheroids, and spheroid-derived hMSCs were seeded at equal cell density (200,000 cells/well or 8–25k spheroids/well) on tissue-culture treated six-well dishes in 1.5 mL of CCM. In addition, hMSC spheroids were also suspended at 8 spheroids/well on nonadherent six-well dishes (Corning). After 24 h, images were acquired, conditioned medium was collected, and the cells were lyzed with 100 μL of modified RIPA buffer containing HALT protease/phosphatase inhibitors (Thermo Scientific). Conditioned medium was cleared of cellular material by centrifugation at 500 × *g* for 10 min and stored at −80 °C. Total cellular protein was measured in whole cells lysates using the bicinchoninic acid (BCA)-dependent colorimetric detection method (Micro BCA Protein Assay Kit; Thermo Scientific). Human TSG-6 protein levels in conditioned medium were determined by ELISA as described (1) with some modifications. Briefly, wells of microplate strips (Costar) were coated overnight at 4 °C with 10 μg/mL TSG-6-specific monoclonal antibody (clone A38.1.20; Santa Cruz Biotechnology, Inc.) in 50 μL of 0.2 M bicarbonate buffer (pH 9.2). The plates were washed 3 times with 400 μL of 1× wash buffer (R&D Systems), blocked with 100 μL of 1× PBST (Cell Signaling) containing 0.5% BSA (Thermo), and incubated for 2 h with 50 μL of sample or recombinant human TSG-6 protein standards (R&D Systems) diluted in blocking buffer. Wells were subsequently washed and incubated with 0.5 μg/mL biotinylated antihuman TSG-6 (R&D Systems) in 50 μL of 1× PBST. After 2 h, the samples were incubated for 20 min with 50 μL of streptavidin-HRP (R&D Systems), then with 100 μL substrate solution (R&D Systems). The colorimetric reaction was terminated after 15 min with 2 N sulfuric acid (R&D Systems) and the optical density determined on a plate reader (FLUOstar Omega; BMG Labtech) at an absorbance of 450 nm with wavelength correction at 540 nm. Human STC-1 and LIF proteins were detected with commercially available ELISA kits (R&D Systems) following procedures described by the manufacturer. Two hundred μL of sample/well was used for the LIF ELISA and 100 μL for the STC-1 ELISA. The obtained values were normalized to total cellular protein content to account for loss of cell/spheroid transfer.

Macrophage Inflammatory Assay. J774A.1 mouse macrophages (ATCC) were cultured on 15-cm bacteriological dishes (Falcon) in high glucose DMEM (Invitrogen) supplemented with glutamax, 10% FBS, and penicillin/streptomycin. Subcultures were prepared by washing the cells from the dish every 2–3 d and replating at a ratio of 1:5 to 1:10. For the inflammatory assay, macrophages (MΦ) were seeded in the upper chamber of a 24-mm transwell insert with 0.4-μm pores (Corning) at 400,000 cells/well followed by stimulation with 0.1 μg/mL of LPS (Sigma). After 90 min, LPS was removed and the cultures replaced with fresh medium. Total of 200,000 monolayer hMSCs, 200,000 spheroid derived cells, or 8–25k spheroids were transferred to the plate beneath the transwell. After 5 h, medium conditioned by the macrophages was collected and clarified by centrifugation at 500 × *g* for 10 min. A total of 50 μL of conditioned medium was used for mTNFα ELISA (Quantikine Kit; R&D Systems). Mouse macrophages were washed with PBS and harvested for RNA to quantify mTNFα expression levels by Real-time RT PCR using Taqman Gene Expression Assay.

Mouse Model of Peritonitis. Male C57BL/6J mice (Jackson Laboratories), 6–8 wk of age and housed on a 12 h light/dark cycle, were used to study the antiinflammatory action of hMSC spheroids on zymosan-induced peritonitis. All animal procedures were performed with approval by the Animal Care and Use Committee of Texas A&M Health Science Center and in accordance with guidelines set forth by the National Institutes of Health. The inflammatory compound Zymosan A (Sigma) was prepared at a concentration of 1 mg/mL in PBS and autoclaved for 15 min to sterilize. To induce inflammation, 1 mL of the 0.1% zymosan solution was administered i.p. Fifteen minutes later, either 1.5×10^6 monolayer hMSCs, 1.5×10^6 spheroid derived cells, or 60–25k spheroids were administered i.p. through a 20-gauge needle or catheter in 150–200 μL of HBSS (Gibco). After 6 h, animals were killed by cervical dislocation and the exudates retrieved by peritoneal lavage using 1.5 mL sterile PBS, pH 7.4, containing a 1× concentration of Halt protease inhibitors (Thermo Scientific) and 5 mM EDTA (Thermo Scientific). The lavage volume was recorded and the cells removed by centrifugation at 500 × *g* for 10 min. The protein-rich supernatants were then transferred to fresh microcentrifuge tubes, cleared of debris by centrifugation for 10 min at 10,000 × *g*, and stored at −80 °C. Amounts of peritoneal exudation were determined by subtracting the measured lavage volume of each sample from the averaged baseline volume. Twenty-four hours after cell delivery, blood was collected from the right ventricle of anesthetized mice and transferred to Capiject clot activating tubes (Terumo Medical Corporation). The tubes were inverted 8–10 times and incubated at room temperature for 20–30 min to facilitate clot formation. The samples were centrifuged at 1500 × *g* for 10 min and the serum layer collected for measurements of plasmin activity.

Measurements of Inflammation in Peritoneal Exudates and Blood Serum. Levels of inflammatory molecules TNFα, IL-1β, CXCL2/MIP-2, and PGE_2 were determined from the peritoneal lavage using commercially available ELISA kits (R&D Systems). Fifty microliters of sample per well was used for the detection of TNFα, 25 μL for IL-1β (1:2 dilution), 6.7 μL for CXCL2 (1:7.5 dilution), and 3 μL for PGE_2 (1:50 dilution). Secreted myeloperoxidase (MPO), a marker of neutrophil activity, was measured from the cell-free lavage fluid with a mouse-specific MPO ELISA kit (Hycult Biotech) per manufacturer's instructions. Total protein was evaluated with the Micro BCA protein detection kit (Thermo Scientific). Serum plasmin activity, a marker of inflammatory status, was ascertained by measuring time dependent cleavage of the chromogenic substrate Chromozym PL (Roche Applied Science) into 4-nitranline in 50 mM Tris-HCl (pH 7.4) and 0.9% NaCl.

Absorbance at 405 nm was measured every 2 min for 30 min using the plate reader. The values were expressed as average change in absorbance/min.

Data Analysis. Data are summarized as mean ± SD. Student's *t* test was used to calculate the levels of significance (NS, $P \geq 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

1. Lee RH, et al. (2009) Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 5:54–63.

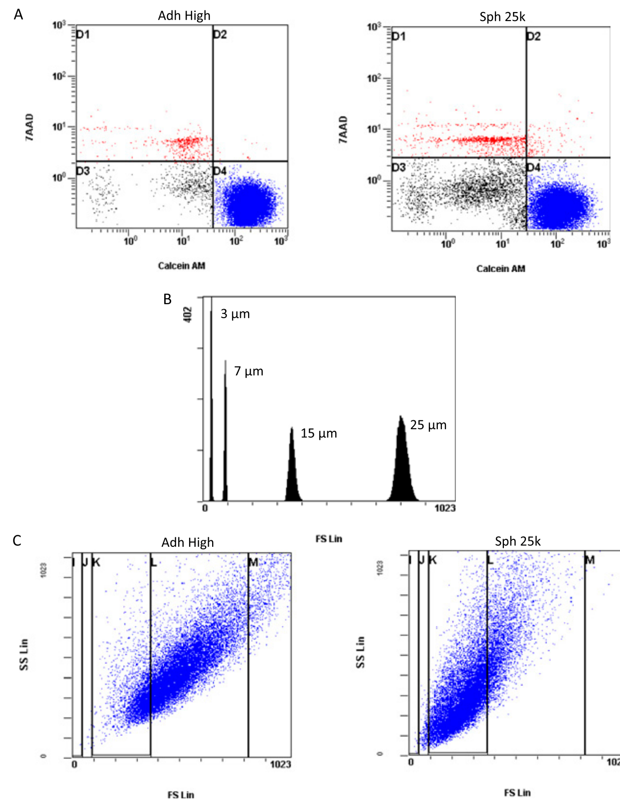

Fig. S1. Analysis of spheroid-derived hMSC size by flow cytometry. Flow cytometric determination of hMSC size from 3-d cultures of adherent monolayers (Adh High) or spheroids (Sph 25k) labeled with the viability dyes calcein AM (live cells, blue) and 7AAD (dead cells, red). (*A*) Representative log fluorescent dot plots. (*B*) Histogram of bead standards with diameters of 3, 7, 15, and 25 µm. (*C*) Representative linear scatter plots of the calcein AM^{+}/$7AAD^{-}$ cell populations. Brackets were applied to the scatter plot at locations corresponding to the appropriate bead size (I = 0, J = 3 µm, K = 7 µm, L = 15 µm, and M = 25 µm). Assays were performed at the same voltages.

Fig. S2. hMSCs from spheroids exhibit similar growth characteristics and clonogenicity to monolayer cultures. (*A*) Growth of hMSCs (donor 1) as monolayers from high density and hanging drop cultures plated at low density (5,500 cells/plate) and passaged every 7 d. Cumulative population doublings (PDs) after each passage are shown (*Inset*). (*B*) CFU-F assays of hMSCs (donor 1) plated at 83 cells/plate and incubated for 14 d. Representative plates at passage 1 and passage 2 after transfer. Values are mean CFU-F percentage ± SD (n = 4).

Fig. S3. Surface phenotype of hMSCs cultured as monolayers at low cell density. Flow cytometry measurements of characteristic hMSC surface proteins. Passage 2 hMSCs were plated at 100 cells/cm^2 and grown for 7 d until approximately 70% confluent before analysis. The cells were harvested by incubation with trypsin/EDTA for 5 min.

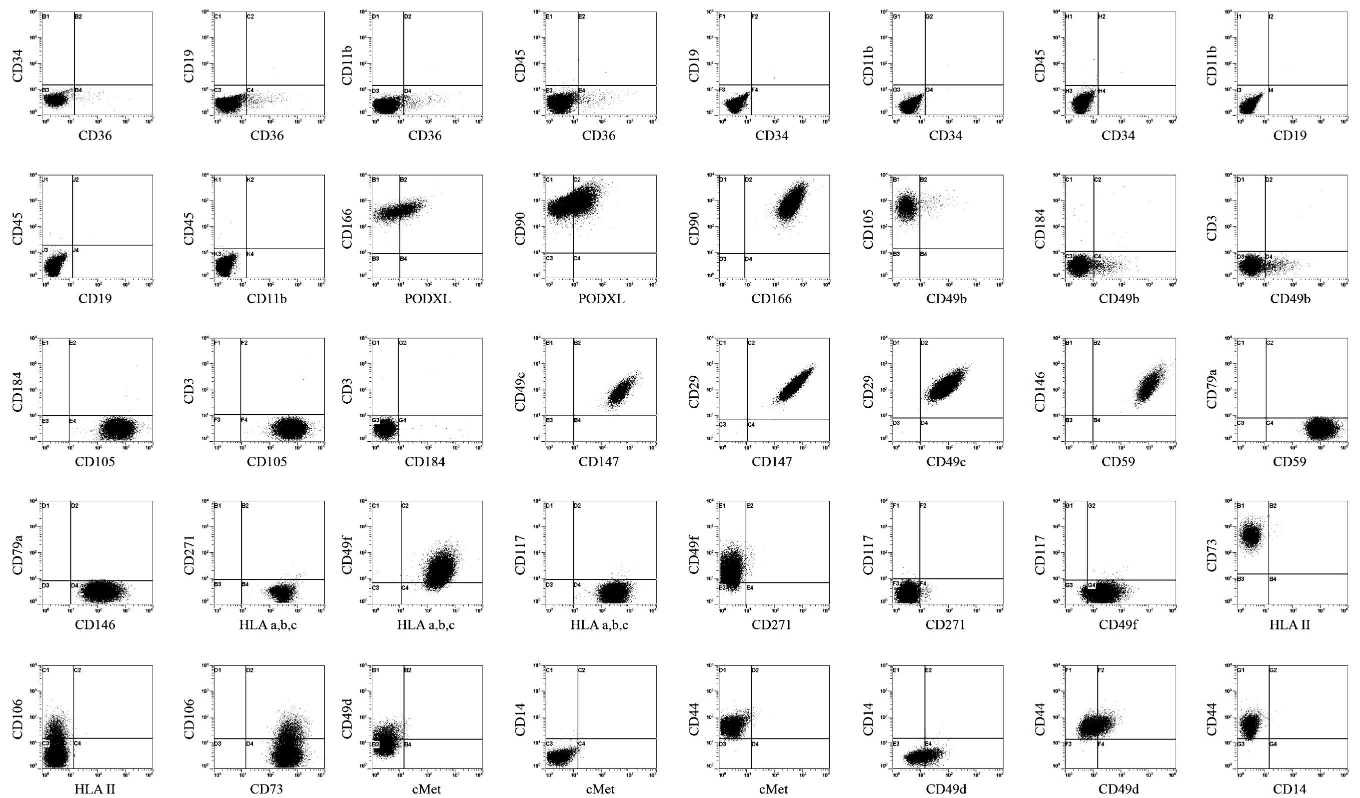

Fig. S4. Surface phenotype of hMSCs cultured as monolayers at high cell density and harvested by incubation with trypsin for 5 min. Flow cytometry measurements of characteristic hMSC surface proteins. Passage 3 hMSCs were plated at 5,000 cells/cm^2 and grown for 3 d before analysis. The cells were harvested by incubation with trypsin/EDTA for 5 min.

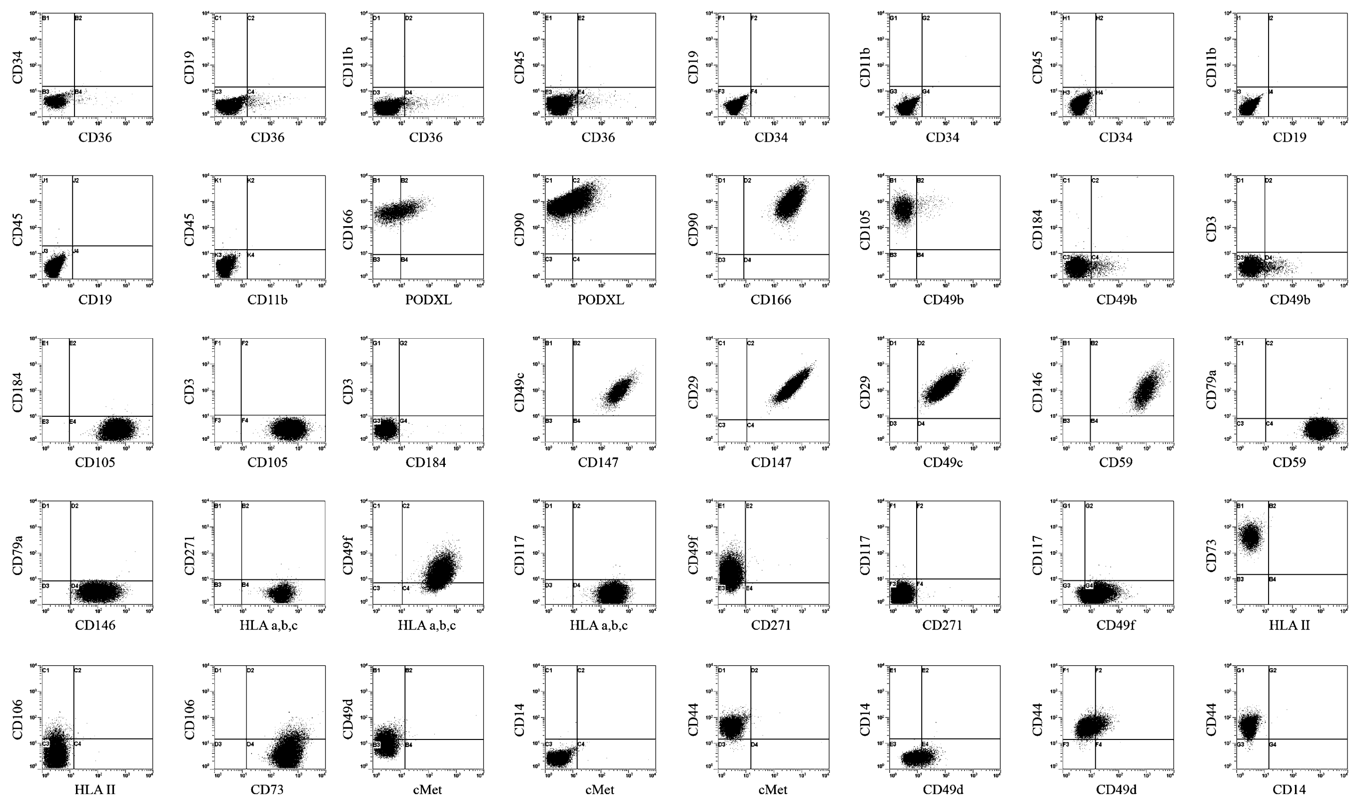

Fig. S5. Surface phenotype of hMSCs cultured as monolayers at high cell density and harvested by incubation with trypsin for 10 min. Flow cytometry measurements of characteristic hMSC surface proteins. Passage 3 hMSCs were plated at 5,000 cells/cm^2 and grown for 3 d before analysis. The cells were harvested by incubation with trypsin/EDTA for 10 min.

Fig. S6. Surface phenotype of hMSCs derived from spheroids. Flow cytometry measurements of characteristic hMSC surface proteins. Passage 3 hMSCs were suspended in hanging drops at 25,000 cells/drop and cultured for 3 d before analysis. The spheroids were dissociated for 10 min with trypsin/EDTA to obtain the spheroid cells.

Fig. S7. Cell cycle analysis of monolayer and spheroid hMSCs. Representative cell cycle distribution in hMSCs for donor 1 grown at low density (*A*), at high density (*B*), and as spheroids (*C*) was determined by analyzing DNA content of permeabilized hMSCs labeled with PI. DNA content was measured with a flow cytometer and data analyzed using MultiCycle software. (*D*) Summary of the cell cycle analysis data. Values are mean ± SD ($n = 3$).

Fig. S8. hMSC spheroids show increased antiinflammatory effects in vitro and in vivo. (*A*) Real-time RT-PCR assays of RNA for mouse TNFα in mouse macrophages (MΦs). MΦs were seeded in the upper chamber of a transwell, stimulated with LPS for 90 min, the LPS was removed, and the chamber transferred to a six-well dish plated with monolayer (Adh), spheroid (Sph), or spheroid derived (Sph DC) hMSCs at equal cell density. MΦ:hMSC (2:1). After 5 h, MΦs were lyzed, RNA was isolated, and mTNFα expression was determined with real-time RT PCR. Values are mean ± SD (n = 3) normalized to MΦ sample without LPS stimulation. (*B–E*) Antiinflammatory activity of hMSCs in a mouse model of peritonitis. C57BL/6 mice were injected i.p. with zymosan to induce inflammation. After 15 min, the mice were injected i.p. with 1.5×10^6 monolayer hMSCs (Adh), 60 spheroids (Sph), or 1.5×10^6 spheroid derived cells (Sph DC). After 6 h, peritoneal lavage was collected and lavage protein content (*B*), volume (*C*), mIL-1β (*D*), and mCXCL2 were determined. Values are mean ± SD (negative control, n = 4; vehicle control, n = 6; Adh treated, n = 6; Sph treated, n = 8; Sph DC treated, n = 6). Not significant (NS) $P \geq 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Table S1. List of antibodies used to detect the expression of cell surface proteins in hMSCs

Protocol	Protein	Fluorochrome	Isotype	Clone	Manufacturer
Protocol 1	CD36	FITC	Ms IgG-1	FA6.152	Beckman-Coulter
	CD34	PE	Ms IgG-1	581	Beckman-Coulter
	CD19	ECD	Ms IgG-1	J3.119	Beckman-Coulter
	CD11b	PE-Cy5	Ms IgG-1	Bear1	Beckman-Coulter
	CD45	PE-Cy7	Ms IgG-1	J.33	Beckman-Coulter
Protocol 2	PCLP1	FITC	Ms IgG-2a	53D11	MBL International
	CD166	PE	Ms IgG-1	3A6	Beckman-Coulter
	CD90	PE-Cy5	Ms IgG-1	Thy1/310	Beckman-Coulter
Protocol 3	CD49b	FITC	Ms IgG-1	Gi9	Beckman-Coulter
	CD105	PE	Ms IgG-3	IG2	Beckman-Coulter
	CD184	APC	Ms IgG-2a	12G5	BD Biosciences
	CD3	PE-Cy7	Ms IgG-1	UCHT1	Beckman-Coulter
Protocol 4	CD147	FITC	Ms IgG-1	HIM6	BD Biosciences
	CD49c	PE	Ms IgG-1	C3 II.1	BD Biosciences
	CD29	PE-Cy5	Ms IgG-1	MAR4	BD Biosciences
Protocol 5	CD59	FITC	Ms IgG-2a	P282E	Beckman-Coulter
	CD146	PE	Ms IgG-2a	TEA1/34	Beckman-Coulter
	CD79a	PE-Cy5	Ms IgG-1	HM47	Beckman-Coulter
Protocol 6	Class I HLA	FITC	Ms IgG-1	G46-2.6	BD Biosciences
	CD271	PE	Ms IgG-1	C40-1457	BD Biosciences
	CD49f	PE-Cy5	Rat IgG-2a	GoH3	BD Biosciences
	CD117	PE-Cy7	Ms IgG-1	104D2D1	Beckman-Coulter
Protocol 7	Class II HLA	FITC	Ms IgG-2a	TU39	BD Biosciences
	CD73	PE	Ms IgG-1	AD2	BD Biosciences
	CD106	PE-Cy5	Ms IgG-1	51–10C9	BD Biosciences
Protocol 8	HGFR	FITC	Rat IgG-1	eBioclone97	eBioscience
	CD49d	PE	Ms IgG-1	9F10	BD Biosciences
	CD14	ECD	Ms IgG-2a	RMO52	Beckman-Coulter
	CD44	APC	Ms IgG-2b	G44-26	BD Biosciences
Protocol 9	Isotypes	FITC	Ms IgG-1	679.1Mc7	Beckman-Coulter
		PE	Ms IgG-2a	7T4-1FS	Beckman-Coulter
		FITC	Ms IgG-2a	G155-178	BD Biosciences
		FITC	Rat IgG-1, *k*		eBioscience
		PE	Ms IgG-1, *k*	MOPC-31C	BD Biosciences
		PE	Ms IgG-3		Santa Cruz
		ECD	Ms IgG-1	679.1MC7	Beckman-Coulter
		ECD	Ms IgG -2a	7T4-1F5	Beckman-Coulter
		PE-Cy5	Ms IgG-1	679.1Mc7	Beckman-Coulter
		PE-Cy5	Rat IgG-2a	R35-95	BD Biosciences
		APC	Ms IgG-2a	7T4-1F5	Beckman-Coulter
		APC	Ms IgG-2b	27–35	BD Biosciences
		PE-Cy7	Ms IgG-1	679.1Mc7	Beckman-Coulter

Total of nine different protocols were run for each hMSC sample to determine the surface proteins expressed.

Table S2. Selected genes up-regulated in hMSC spheroids

Gene (GeneID)	Donor 1		Donor 2	
	25k Sph vs. Adh Low	25k Sph vs. Adh High	25k Sph vs. Adh Low	25k Sph vs. Adh High
Secreted molecules				
IL8 (3576)	78	82	38	34
TSG-6 (7130)	55	61	51	40
IL1B (3553)	3	24	19	12
BMP2 (650)	16	14	23	12
CXCL1 (2919)	14	12	7	3
SPP1 (6696)	13	12	4	5
GDF15 (9518)	12	6	17	6
IL11 (3589)	11	10	9	10
LIF (3976)	10	12	7	9
SMOC1 (64093)	10	8	6	4
IL1A (3552)	9	7	5	3
IGFBP5 (3488)	8	11	11	14
C1S (716)	8	4	9	3
BMP6 (654)	7	8	3	4
TRAIL (8743)	7	7	11	6
PTHLH (5744)	6	6	3	3
NMB (4828)	6	5	5	3
APOD (347)	6	5	7	3
PLTP (5360)	6	7	5	4
IL24 (11009)	6	6	10	7
IL6 (3569)	6	3	3	3
STC1 (6781)	6	7	6	10
NAMPT (10135)	5	5	3	3
Cell surface receptors				
ITGA2 (3673)	26	23	13	18
EDNRA (1909)	21	15	7	9
GPR84 (53831)	18	13	11	5
BDKRB2 (624)	10	10	9	6
CXCR4 (7852)	7	7	4	5
DPP4 (1803)	7	6	5	4
CD82 (3732)	6	5	7	4
PLA2R1 (22925)	6	5	7	4
PTGDR (5729)	6	7	7	5
ICAM1 (3383)	5	6	8	5
COLEC12 (81035)	5	4	7	6
C3AR1 (719)	5	5	4	3
Extracellular matrix molecules				
MMP13 (4322)	64	66	39	37
CHI3L1 (1116)	42	33	72	36
TFPI2 (7980)	25	55	15	53
MMP3 (4314)	14	15	9	6
MMP1 (4312)	10	11	25	16
ADAMTS5 (11096)	8	7	5	3
GPC6 (10082)	6	4	3	2
LUM (4060)	6	3	6	3
LAMA4 (3910)	5	3	5	3
Transcription factors				
NR4A2 (4929)	11	12	13	10
ETV1 (2115)	10	11	6	6
MAFB (9935)	9	9	6	6
SATB1 (6304)	6	6	7	5

Values are fold changes of 25k Sph sample compared with either Adh Low or Adh High for two donors.